

## Epitope Mapping of Anti-recA Protein IgGs by Region Specified Polymerase Chain Reaction Mutagenesis\*

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Monoclonal IgGs were shown to be useful for the specific inhibition of a set of activities of the recA protein, a key protein in homologous genetic recombination. The mapping of the epitopes for these IgGs and site-directed mutagenesis based on the mapping will facilitate location of the functionally active sites on the tertiary structure of the protein, which is being solved by means of physicochemical techniques. We developed a novel technique for region-specified mutagenesis and applied the technique to epitope mapping. Using the polymerase chain reaction in the presence of deoxyinosine triphosphate, we introduced random base substitutions specifically into a region of the *recA* gene defined by a pair of primers. RecA mutants exhibiting altered antigenicity were selected, in plaque-immunoblotting experiments, from libraries of mutagenized *recA* genes constructed on the  $\lambda$ gt11 expression vector. Mutant *recA* genes were obtained at the frequency of about  $10^{-2}$  among the plaques expressing fused *recA* genes and then each one was expressed as a whole protein, which was characterized by enzyme-linked immunosorbent assay. Analyzing the DNA sequences of the mutant *recA* genes, we located at the amino acid sequence level the epitopes for two anti-recA IgGs which could not be located in previous studies. One of the antibodies was shown to prevent self-assembly of the recA protein and the other was suggested to inhibit the binding of double-stranded DNA. Thus, the active sites involved in these functions would be located in the space around or near the relevant epitope.

The recA protein, and its prokaryotic and virus (T4 phage) analogues promote "homologous pairing" and "strand exchange" between homologous double-stranded and single-stranded DNAs through ATP (or dATP)-dependent reactions *in vitro*, and were shown to play an essential role in homologous genetic recombination *in vivo*. Homologous pairing is the formation of an intermolecular duplex ("heteroduplex") between a couple of homologous single-stranded and double-stranded DNAs, and strand exchange is the processing of the heteroduplex, such as its elongation. Each of these reactions consist of a number of substeps and the recA protein or its analogues appear to have various active sites that

promote each of these substeps, such as an ATP-binding site, ATPase catalytic center, binding site for single-stranded DNA, binding site for double-stranded DNA, and sites for self-polymerization. The localization of these active sites on the tertiary structure of the recA protein is essential for understanding the mechanisms of the underlying biochemical functions of the protein. However, only sites related to ATPase have been partly located at the amino acid sequence level. The mapping of mutation sites as well as x-ray crystallographic analysis of the protein are the main means to this end. A series of our studies involving the use of anti-recA protein monoclonal IgGs is also an approach to the same goal (see Shibata *et al.*, 1991, for review).

We have isolated clones of mouse hybridomas which produce anti-recA protein IgGs (Makino *et al.*, 1985). Two (ARM193 and ARM191) of these anti-recA protein IgGs each inhibit a set of activities of the recA protein without affecting the others; i.e. ARM193 severely inhibits the unwinding of the double helix and strand exchange, but allows homologous pairing and single-stranded DNA-dependent ATP hydrolysis (Ikawa *et al.*, 1989; Makino *et al.*, 1985, 1987). On the other hand, ARM191 inhibits the homologous pairing and unwinding of the double helix, but only affects the single-stranded DNA-dependent ATP hydrolysis a little (Makino *et al.*, 1985). ARM193 was suggested to affect the site for the interaction between recA polypeptides (Ikawa *et al.*, 1989) and ARM191 to affect the site on the recA polypeptide for the binding to double-stranded DNA (Makino *et al.*, 1985). We preliminarily located the epitopes for both ARM193 and ARM191 in a C-terminal 88 amino acid region (Phe<sup>260</sup>-Glu<sup>347</sup>) of the recA polypeptide by examining the cross-reaction of proteolytic fragments. However, we failed to map them more precisely, since none of the subfragments of the 88-amino acid region exhibited significant cross-reaction with either of the IgGs (Ikeda *et al.*, 1990). Therefore, it was necessary to introduce another technique to overcome the problem. Here, we describe a novel technique for region-specified mutagenesis and, as an application of this technique, the mapping of the epitopes of ARM193 and ARM191 at the amino acid sequence level in distinct but slightly overlapped subregions in the C-terminal 88-amino acid region.

### MATERIALS AND METHODS

**recA Protein**—The purified recA protein was fraction V prepared as described (Shibata *et al.* 1981).

**Oligonucleotides**—Oligonucleotides were synthesized with a DNA synthesizer (Du Pont-New England Nuclear CODER300) and purified with NENSORB PREP (Du Pont-New England Nuclear).

**Techniques for Cloning of DNAs**—Treatment with restriction endonucleases and DNA ligase, and the isolation and cloning of the DNA fragments onto vectors were carried out as described (Berger and Kimmel, 1987; Maniatis *et al.*, 1982).

**Antibodies and Immunochemical Techniques**—The anti-recA pro-

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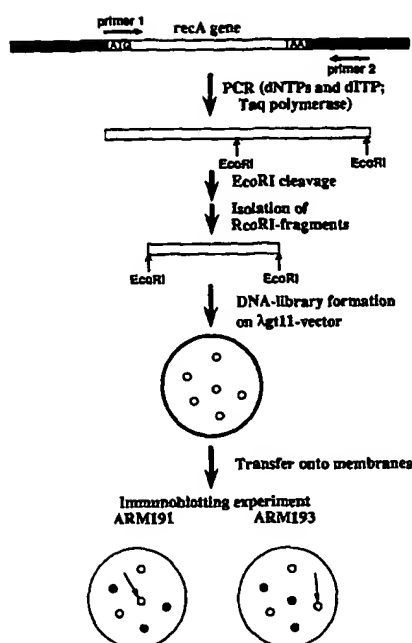


FIG. 1. Region-specified PCR mutagenesis. DNA encoding the *E. coli recA* gene flanked by primers 1 and 2 was amplified by PCR in the presence of dITP with the use of *Taq* DNA polymerase. *EcoRI* fragments of the amplified DNA which encoded the C-terminal region of the *recA* protein were cloned on a  $\lambda$ gt11 expression vector to construct DNA libraries of the mutagenized *recA* genes. With appropriate orientation of a fragment relative to the vector, the C-terminal region of the *recA* gene was connected to the *lacZ* gene in-frame. Proteins expressed in the plaques obtained from the libraries were transferred to a pair of membranes and then the cross-reaction with either anti-*recA* protein IgG ARM191 or ARM193 was tested. The plaques showing cross-reaction with only one of the IgGs (indicated by arrows) were picked up and subjected to further cross-reaction tests. The closed circles in the big circles at the bottom of the figure denote plaques which showed cross-reaction with the indicated IgG.

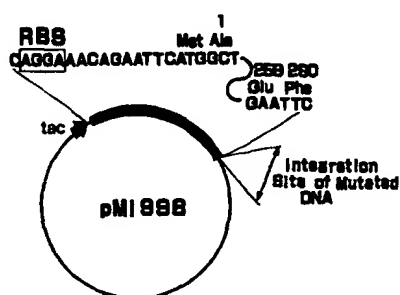


FIG. 2. Structure of pMI996 for expression of mutated *recA* genes. DNA encoding the N-terminal region of the *recA* polypeptide was put under the control of the *Tac* promoter on a multicopy plasmid (pKK223-3). *EcoRI* fragments of the mutagenized *recA* gene were inserted at the *EcoRI* site at the codons for Glu<sup>259</sup>-Phe<sup>260</sup>.

tein monoclonal IgGs, ARM191, ARM193, and ARM414, were described previously. We used affinity purified preparations of these IgGs. An anti-*recA* protein monoclonal IgG, MAbl56, was isolated by Karu and Allen (Karu and Allen, 1982), and a purified preparation of this IgG was provided by Dr. Alexander Karu (University of California, Berkeley) and Dr. A. John Clark (University of California, Berkeley).

The enzyme-linked immunosorbent assay (ELISA)<sup>1</sup> was carried out as described previously. Unless otherwise stated, samples of cell-free extracts were diluted in PBS (50 mM potassium phosphate buffer

<sup>1</sup> The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

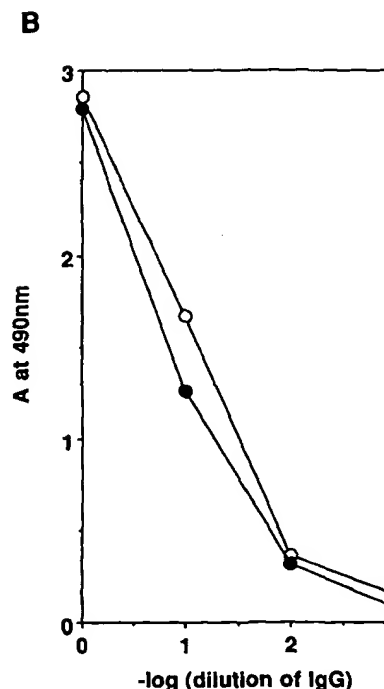
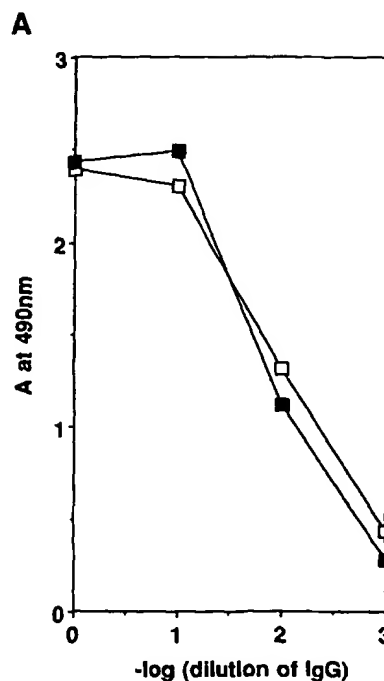


FIG. 3. Absence of competition in cross-reaction between anti-*recA* protein IgGs, ARM191 and ARM193. The wells of a microtiter plate were coated with the purified *recA* protein. Then, a solution of a tested anti-*recA* protein (nondilution: 1.5  $\mu$ g/ml) containing the other IgG was put into the wells and allowed to cross-react with the *recA* protein. ARM191 belongs to IgG<sub>1</sub> and ARM193 to IgG<sub>2b</sub>. Thus, the amounts of ARM191 and ARM193 bound to the *recA* protein were specifically measured by ELISA with the use of an appropriate subclass-specific antibody. A:  $\square$ , the binding of ARM191 in the absence of ARM193;  $\blacksquare$ , the binding of ARM191 in the presence of 5  $\mu$ g of ARM193/ml. B:  $\circ$ , the binding of ARM193 in the absence of ARM191;  $\bullet$ , the binding of ARM193 in the presence of 5  $\mu$ g of ARM191/ml.

(pH 7.2) containing 150 mM NaCl) and put into the sample wells of a microtiter plate with 96 wells. On the other hand, the concentration of the anti-*recA* protein IgG was first adjusted to 30  $\mu$ g of protein/ml ("no dilution") in PBS Tween (PBS containing 0.05% Tween 20) and then a series of dilutions was prepared in PBS/Tween.

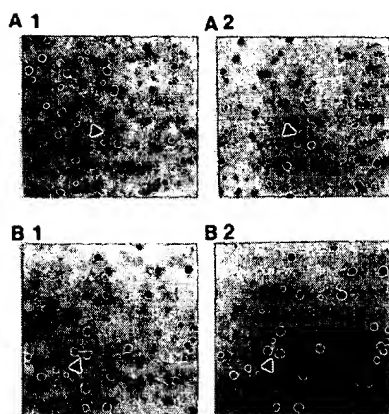


FIG. 4. Testing of cross-reaction of proteins in plaques obtained from libraries of mutagenized DNA. The results of two experiments are shown as examples (A and B). A1 and B1 show cross-reaction with ARM191, and A2 and B2 that with ARM193. The procedure was described in detail in the legend to Fig. 1. Arrowheads indicate mutants which showed altered cross-reaction; mutant D (the top ones in A1 and A2) shows no cross-reaction with ARM191, and mutant 47 (B1 and B2) none with ARM193.

TABLE I  
Isolation of mutants by region-specified PCR mutagenesis

dITP $\mu$ M	Total plaques expressing <i>recA</i> genes	Number of plaques picked up on the first selection	Total number of mutants identified	Number of species of mutants
0	1800	5	1	1
0.2	1100	21	4	2
20	2200	5	3	3
200	2000	21	11	10

**Testing of the Competition between ARM191 and ARM193**—The IgGs, ARM191 and ARM193, belong to subclasses 1 and 2b, respectively (Ikeda *et al.*, 1990), and thus each could be assayed by use of anti-mouse IgG<sub>1</sub> and anti-mouse IgG<sub>2b</sub> antibodies, respectively. A solution of the purified *recA* protein (0.2  $\mu$ g/ml) was put into the wells of a microtiter plate to coat the walls of the wells. Each solution of an indicated amount (no dilution: 1.5  $\mu$ g/ml) of a tested anti-*recA* protein IgG (50  $\mu$ l) contained 0 or 5  $\mu$ g of the other anti-*recA* protein IgG/ml. The amounts of the tested anti-*recA* protein IgG that bound to the wells were measured by ELISA with anti-mouse IgG<sub>1</sub> or anti-mouse IgG<sub>2b</sub> antibodies (Zymed Laboratories, Inc.).

**Region-specified PCR Mutagenesis**—An outline of the procedure is given in Fig. 1. pBEU14 DNA, which contains the *Escherichia coli* *recA* gene (Uhlir and Clark, 1981), was linearized with *Bam*HI and used as the template for PCR (polymerase chain reaction). A DNA region encoding the *recA* gene flanked by primer 1 (5'-ATGGCT-ATCGACGAAAACAA-3') and primer 2 (5'-GAATTCTGTCATG-GCATATCCTT-3') was amplified by 25 cycles of PCR (see Fig. 1). Unless otherwise stated, the reaction mixture for PCR comprised 1  $\mu$ M each of primers 1 and 2, about 3 pM the template DNA, 200  $\mu$ M each of dATP, dTTP, dGTP, and dCTP, 200  $\mu$ M deoxyinosine 5'-triphosphate (dITP), 0.025 units of *Taq* DNA polymerase/ $\mu$ l, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.001% gelatin, and 10 mM Tris-HCl buffer (pH 8.3). Each cycle of PCR consisted of: (i) incubation at 37 °C for 2 min, for loading of the primers onto the template DNA, (ii) incubation at 72 °C for 3 min, for polymerization, and (iii) incubation at 94 °C for 1 min, for denaturation. The amplified DNA was treated with *Eco*RI and the *Eco*RI fragment encoding the C-terminal 93-amino acid region was isolated by gel electrophoresis, followed by trapping on a DEAE membrane (NA45; Schleicher & Schuell). Then, DNA libraries containing the mutagenized *Eco*RI fragments were constructed on the  $\lambda$ gt11 expression vector by ligating them at an *Eco*RI site of the vector. With appropriate orientation of a fragment relative to the vector, the C-terminal region of the *recA* gene was connected to the *lacZ* gene in-frame. Plaques of the phages in the libraries were obtained with the *E. coli* Y1090 strain as a host. Proteins in the plaques were transferred to a pair of membranes and then the cross-reaction with either ARM191 or ARM193 anti-*recA* protein IgG was

examined by means of immunoblotting experiments, as described previously (Ikeda *et al.*, 1990; Morishima *et al.*, 1990). The plaques showing cross-reaction with only one of the IgGs (indicated by arrows in Fig. 1; examples are shown in Fig. 4) were picked up, and the phages were obtained after repeated single plaque isolation and testing by means of immunoblotting experiments.

**DNA Sequence Analysis**—The tested DNAs were cloned on pUC119 and then their sequences were analyzed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977); the labeling reaction was carried out by use of the double-stranded template according to a manual for Sequenase (United States Biochemical Co., Cleveland, OH), and the products were analyzed with the use of an automated DNA sequence analyzer (Du Pont GENESIS2000). We analyzed both strands in most of the cases.

**Expression of Mutant *recA* Genes and Preparation of Cell-free Extracts**—*Eco*RI fragments which carried mutation(s) in the C-terminal 93-amino acid region were ligated to the *Eco*RI site of the DNA region encoding the N-terminal 260 amino acids, which was under the control of the Tac promoter on a multicopy plasmid, pMI996 (Fig. 2), a derivative of pKK223-3 (Brosius and Holy, 1984). An *E. coli* strain, MV1184 (deletion of the *srl-recA* locus) (Vieira and Messing, 1987), was transformed with the DNA. The transformants were grown at 37 °C to the mid-logarithmic growth phase in 3-ml cultures and then expression of the mutated *recA* genes was induced by isopropyl- $\beta$ -D-thiogalactoside treatment (0.2 mg/ml) for 2 h. After the treatment, the cells were collected by centrifugation and suspended in 200  $\mu$ l of a lysis buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 M NaCl, and 5% Triton X-100), and then treated with lysozyme (0.6 mg/ml) at 0 °C for 15 min, followed by the addition of KCl (at 0.24 M). The lysate was centrifuged at about 15,000  $\times g$  for 15 min and the supernatant of the lysate was saved. The supernatant was diluted about 20-fold with PBS and then subjected to ELISA as described above. In the case of mutants 2 and 38, the precipitates of the lysates were resuspended in 5 M urea containing 100 mM Tris-HCl (pH 7.9), followed by centrifugation, and the mutant proteins were extracted from the precipitates with 100 mM Tris-HCl buffer (pH 7.9) containing 8 M urea and 0.1 M NaCl at 25 °C for 1 h.

## RESULTS

**Testing of the Competition between ARM191 and ARM193**—The preliminary mapping of the epitopes of ARM191 and ARM193 indicated that both epitopes were located between Phe<sup>260</sup> and Glu<sup>347</sup> (Ikeda *et al.*, 1990). First, we examined whether or not these anti-*recA* protein-IgGs showed competition in the cross-reaction with the *recA* protein. Since ARM191 and ARM193 belong to different IgG subclasses, each can be discriminated through the use of subclass-specific antibodies on ELISA. Fig. 3 indicates that the presence of one of these IgGs did not affect the binding of the other IgG to the *recA* protein. We conclude that the epitope for ARM191 and ARM193 are different. Thus, we tried to locate the epitopes of these anti-*recA* protein IgGs more precisely.

**Development of Region-specified PCR Mutagenesis and Isolation of Mutants**—Since we were not able to locate the epitopes of ARM191 and ARM193 by examinations of the cross-reaction of subfragments of the *recA* polypeptide, we developed a novel technique for region-specified mutagenesis which could be applied for mapping of the epitopes. The whole process for the isolation of mutants causing altered cross-reaction consists of three stages (Fig. 1); the introduction of region-specified random base substitutions by means of PCR, construction of libraries of the mutagenized DNA with the use of the  $\lambda$ gt11 expression vector, and *in situ* testing for cross-reaction of the mutated polypeptides expressed in the plaques obtained from the libraries. In PCR (Saiki *et al.*, 1985), *Taq* DNA polymerase causes the misincorporation of nucleotides (Eckert and Kunkel, 1990). We added deoxyinosine 5'-triphosphate (dITP), at 200  $\mu$ M, to the reaction mixture for PCR to enhance the misincorporation (Martin and Castro, 1985), and specifically amplified a DNA region defined by a pair of oligonucleotides (primers 1 and 2). Primer 1 includes the initiation codon of the *recA* polypeptide and

Some mutants have base substitutions at the same sites. Except mutants 23 and 34 (substitution of Ser<sup>333</sup>), such overlapped mutants were isolated from the same PCR preparations. Thus, these overlapped mutations seem to be created at an early cycle in PCR and amplified during the procedure.

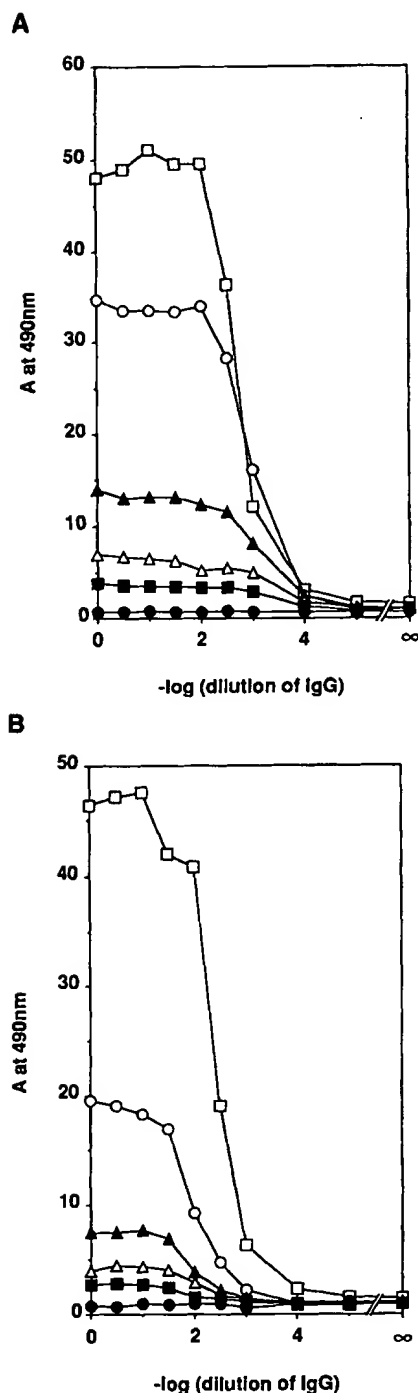


FIG. 5. Variation of the antigen concentration on ELISA does not change the amount of IgG giving a half-maximum signal. The wells of a microtiter plate were coated with the purified *recA* protein at the indicated concentrations. On the other hand, the concentration of the anti-*recA* protein IgG (ARM191 in A; ARM193 in B) was adjusted to 30 µg/ml ("nondilution"), and then a series of dilutions of the IgG was put into individual wells. The bound IgG was measured by ELISA. The concentrations of the *recA* protein were as follows: □, 2 µg/ml; ○, 0.2 µg/ml; ▲, 0.1 µg/ml; △, 0.05 µg/ml; ■, 0.02 µg/ml; ●, without *recA* protein.

Except for in these cases, base substitutions appeared to be introduced at random in the amplified DNA.

**Cross-reaction of Mutant *recA* Proteins**—We constructed a plasmid (pMI996; Fig. 2) in which the wild type *recA* gene was under the control of the *Tac* promoter on a multicopy plasmid (a derivative of pKK223-3 (Brosius and Holy, 1984). We replaced the *EcoRI-EcoRI* region encoding the C-terminal

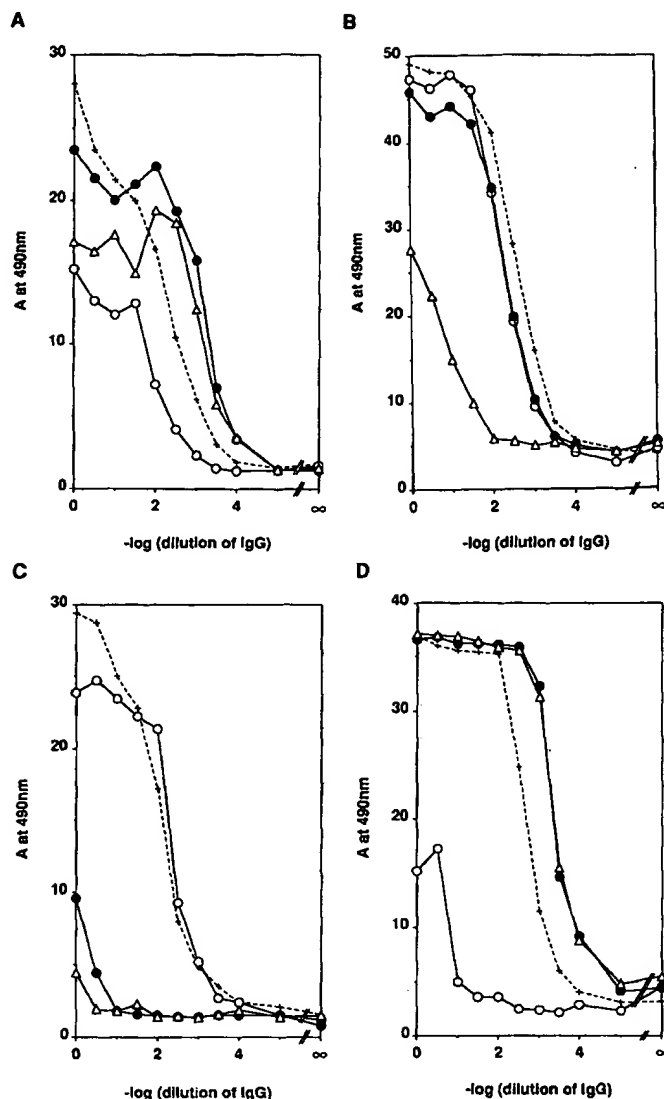


FIG. 6. Examples of ELISA for testing the cross-reaction of mutant *recA* proteins to anti-*recA* protein IgGs, ARM191, ARM193, and Mab156. Cell-free extracts were prepared from cells in which the mutant *recA* genes were expressed; A, wild type; B, mutant 38; C, mutant 32; D, mutant 31. The cell-free extracts were put into individual wells of a microtiter plate to allow the adsorption of proteins. Then, the cross-reaction to each of the IgGs was examined by ELISA, as described in the legend to Fig. 5. The IgGs used were: ●, ARM191; ○, ARM193; △, Mab156. Anti-*recA* protein IgG, ARM414, of which epitopes is located between Glu<sup>233</sup> and Lys<sup>256</sup> (Ikeda *et al.*, 1990) was used as a positive control (+).

93 amino acids of the wild type *recA* polypeptide with the *EcoRI* fragments on which we found mutation(s) (Fig. 2). Then, a mutant of *E. coli* in which the whole *recA* gene was deleted was transformed with these plasmids. The expression of the mutant *recA* genes was induced and cell-free extracts were prepared, followed by quantitative assaying (ELISA) for cross-reaction with ARM191 and ARM193. For comparison of the extent of cross-reaction of a tested IgG with the *recA* protein, we determined the amount of the IgG giving a half-maximum signal on ELISA. As shown in Fig. 5, variations in the amount of *recA* protein did not significantly change the amount of the IgG which gave the half-maximum signal on ELISA. We calculated the amounts of ARM191 and ARM193 giving a half-maximum signal to be 0.0277 ( $\sigma = 0.017$ ) and 0.29 ( $\sigma = 0.10$ ) µg/ml, respectively, from the data in this figure.

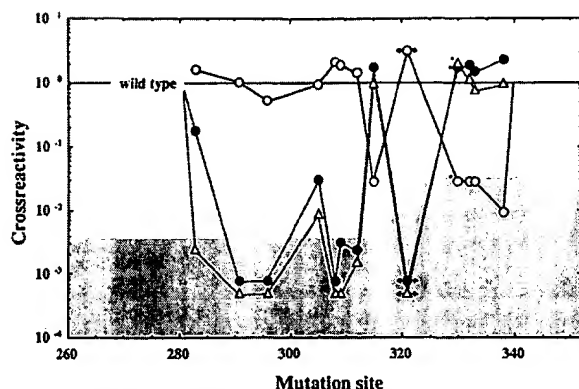


FIG. 7. Mutation sites and alterations in cross-reactivity. The numbers under the abscissa are the amino acid positions from the N terminus of the *recA* polypeptide. Cross-reactivity is defined as follows: (the amount of an IgG giving the half-maximum value on ELISA with wild-type *recA* protein)/(the amount of an IgG giving the half-maximum value on ELISA with mutant *recA* protein). Thus, it shows the deficiency in the cross-reaction. ●, cross-reaction with ARM191; ○, that with ARM193; △, that with MAb156. Symbols within the shadowed area indicate that no cross-reaction was detected (within the limit of the measurement, which is indicated by each symbol). The symbols with a horizontal arrow around position 320 are those for a mutant having two amino acid substitutions at positions 319 and 323, and those with \* at position 330 are those for a mutant having two amino acid substitutions at positions 294 and 330.

Examples of ELISA with mutant *recA* proteins as well as the wild type protein are shown in Fig. 6. The values calculated on ELISA are listed in Table II. In order to obtain the half-maximum signals on ELISA, all proteins (except mutant 38) which had been determined to be mutants by immunoblotting experiments were shown to require at least 10-fold more IgG ARM191 or ARM193 than the average for plus proteins (Fig. 7). The results shown in Fig. 7 clearly indicate that the mutants affecting the cross-reaction with ARM191 and those affecting that with ARM193 are separately clustered, and only slightly overlap each other (between positions 315 and about 320). This conclusion is consistent with the absence of competition in the binding of these IgGs to the *recA* protein (Fig. 3).

**Comparison of MAb156 with ARM191 and ARM193—**MAb156 was isolated by Karu and Allen (Karu and Allen, 1982). Since it was assumed that the epitope of this antibody is located near the C terminus of the *recA* polypeptide,<sup>2</sup> we examined the cross-reaction of MAb156 with the mutant *recA* proteins isolated in the present study. All of the mutations affecting the cross-reaction with ARM191 also affected the cross-reaction with MAb156, but none of the mutations affecting the cross-reaction with ARM193 did. This suggests that the epitope for MAb156 and that of ARM191 are similar, but the following results show that they are not identical. Most of the mutations abolishing the cross-reaction with ARM191 also abolished that with MAb156 (Fig. 7), but substitution of Leu<sup>283</sup> by Pro in mutant 38 strongly interfered with the cross-reaction with MAb156, but not so much with that with ARM191 (Figs. 6B and 7). The difference in the mode of cross-reaction between ARM191 and MAb156 was also observed with a mutant *recA* protein having a substitution of Ala<sup>305</sup> by Gly (Fig. 7).

#### DISCUSSION

Region-specific PCR mutagenesis is an efficient tool for introducing random base substitution mutations specifically

in a region defined by a pair of primers. From among the plaques expressing the fused *recA* gene, we obtained mutants of the *recA* gene at the frequency of 0.5% under the optimized conditions. Using this mutagenesis, we identified regions of the *recA* polypeptide in which amino acid substitutions prevent the cross-reaction with ARM191, ARM193, and/or MAb156. The region for ARM191 and that for ARM193 are different, but slightly overlap each other; i.e. that for the former IgG comprises positions 283 through about 320, and that for the latter positions through 315–338. Since ARM191 and ARM193 showed no competition, Thr<sup>315</sup>, and Ile<sup>319</sup> and Ile<sup>319</sup> and/or Val<sup>323</sup> would be located on different sides of a local structure or the whole molecule of the *recA* polypeptide.

The substitution of an amino acid might interfere with the cross-reaction with an IgG directly or through a very local change in the tertiary structure, or indirectly through extensive alteration of the tertiary structure of the *recA* protein. The Leu<sup>283</sup> to Pro substitution in mutant 38 could cause gross alteration of the tertiary structure of the *recA* protein. The clustering of other amino acid substitutions in a particular region is favorable for the first two possibilities rather than the last one. ARM193 was shown to prevent the self-assembly of the *recA* protein and ARM191 to inhibit the binding of double-stranded DNA. Thus, the active sites involved in these functions would be located in the space at or around the relevant epitope. We are testing these possibilities by examining the effects of the mutant *recA* proteins isolated in this study and those constructed by another round of region-specified PCR mutagenesis. Determination of the epitope loci and the inhibitory effects of these IgGs, together with the tertiary structure of the *recA* protein, will facilitate the understanding of the function of the protein in relation to its structure.

The technique of region-specified random base substitutions involving the use of PCR employed in this study is very useful not only for epitope mapping, as described in this paper, but is also widely useful for studies on the function of a gene and an enzyme or protein, because of the flexibility as to specifying a target region, and the high yield of the random base substitutions.

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<sup>2</sup> A. J. Clark and A. Karu, personal communication.